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Biocontrol efficacy and other characteristics of protoplast fusants between *Trichoderma koningii* and *T. virens*

Linda E. HANSON[1]* and Charles R. HOWELL[2]

[1] *USDA-ARS Sugar Beet Research Unit, Crops Research Laboratory, 1701 Centre Avenue, Fort Collins, CO 80526, USA.*
[2] *USDA-ARS SPARC, Cotton Pathology Research Unit, 2765 F&B Road, College Station, TX 77845, USA.*
E-mail: *lehanson@lamar.colostate.edu*

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Several *Trichoderma virens* (syn. *Gliocladium virens*) strains have good biocontrol activity against *Rhizoctonia solani* on cotton but lack some of the commercially desirable characteristics found in other *Trichoderma* species. In an attempt to combine these desirable characteristics, we used a highly effective biocontrol strain of *T. virens* in protoplast fusions with a strain of *T. koningii*, which had good storage qualities, but little biocontrol efficacy. All fusants were morphologically similar to one of the parental species. However, when compared to the morphologically similar *T. koningii* parent, two fusants showed significantly better biocontrol activity against *R. solani* on cotton. In addition, one *T. virens*-like fusant gave significantly less control than the *T. virens* parent. Fusants also differed from the morphologically similar parent in the production of secondary metabolites. One fusant was obtained which maintained biocontrol activity during storage for up to a year.

INTRODUCTION

Biocontrol of plant diseases offers many benefits in disease management. For example, biocontrol agents can control diseases for which there is no effective chemical control or host resistance, and may be of less environmental concern than traditional chemical pesticides (Becker & Schwinn 1993).

Trichoderma species exhibit biocontrol activity against a number of different fungi on various hosts (Papavizas 1985). Several strains of *Trichoderma virens* (syn. *Gliocladium virens*) have been found that show biocontrol activity (Lewis, Papavizas & Hollenbeck 1993), including activity against *Rhizoctonia solani* on cotton (Howell 1982) and other crops (Lumsden & Locke 1989, Yuen, Craig & Giesler 1994). However, these strains may be limited in their utility due to poor maintenance of biocontrol activity in storage at ambient temperatures (Howell 1991). Storage limitations are one of the factors that must be addressed to ensure that biocontrol agents are commercially viable (Becker & Schwinn 1993). This limitation might be overcome by incorporation of good storage characters from another organism.

One method of combining characteristics from different fungi is protoplast fusion. Protoplast fusion allows the transfer of complex traits without having to know the genes involved, and for genetic recombination between organisms that cannot undergo sexual recombination (Pe'er & Chet 1990). Protoplast fusions have been performed with *Trichoderma* species for interstrain (Pe'er & Chet 1990, Sivan & Harman 1991, Stasz, Harman & Weeden 1988), interspecific (Stasz, Harman & Gullino 1989), and even intergeneric crosses (Kumari & Panda 1994, Nutsubidze *et al*. 1991). It has been used to combine characteristics such as cellulose decomposition and ethanol production (Kumari & Panda 1994) and to improve rhizosphere competence in *T. harzianum* (Sivan & Harman 1991).

The aim of this research was to make protoplast fusants between a strain of *T. virens* with good biocontrol properties, and a strain of *T. koningii* that retains rapid germination after extended periods in storage, in order to combine desirable characteristics, especially biocontrol activity and maintenance in storage, from the two species.

MATERIALS AND METHODS

Fungal strains

Strains used in this experiment included *Trichoderma virens* strain G6 (ATCC MYA-298), an isolate from Texas cotton field soil with good biocontrol activity (Howell, Stipanovic & Lumsden 1993) and *T. koningii* strain Tk7 (ATCC MYA-648), an isolate from Texas wheat field soil, determined to have good storage properties by preliminary experiments (Howell,

* Corresponding author.

unpubl.). Tk7 was confirmed as morphologically *T. koningii* by Gary J. Samuels (USDA Agricultural Research Service, Beltsville, MD; strain ref. 01-332). All fungi were maintained on potato dextrose agar (PDA) at 28 °C unless stated otherwise.

Protoplast preparation and fusion

For protoplasting, single 4 mm plugs of *Trichoderma koningii* mycelium from 48 h-old PDA cultures or *T. virens* conidia (5×10^3) from 4-d-old cultures were introduced into 100 ml lots of glucose, yeast extract, and casein enzymatic hydrolysate medium (GYEC) (15 g glucose, 3 g yeast extract and 5 g casein enzymatic hydrolysate l^{-1}). Cultures were grown for 24 h at 28 ° on a rotary shaker at 150 rpm. Hyphae of both *Trichoderma* species were harvested separately by vacuum filtration and washed with sterile distilled water. Hyphae (0.6–0.7 g) of each species were suspended in 5 ml of sterile 0.1 M potassium phosphate buffer (pH 6.4) with 0.6 M sorbitol as an osmoticum (KPS) and digested for 3 h at 30 ° with 6 mg ml^{-1} of lysing enzymes from *T. harzianum* (Sigma) and 2 mg ml^{-1} cellulase (Sigma). Protoplasts were harvested by filtration through a 10 μm mesh metal sieve, followed by centrifugation in a microcentrifuge at 100 ***g*** for 10 min at 26 °. Protoplasts were washed and resuspended in KPS. Protoplasts were then counted with a haemocytometer and used immediately or stored for up to 24 h at 4 ° prior to use. No loss in viability was detected with storage for up to 2 d (data not published).

Protoplast fusions were performed using approximately equal numbers of protoplasts of the two species (approximately 10^7 ml^{-1}) in 30% (weight to volume) polyethyleneglycol (PEG, molecular weight 4000) in 0.1 M potassium phosphate buffer (pH 6.4) containing 10 mM $CaCl_2$. Protoplasts were incubated for 30 min at 28 °, collected by centrifugation (as above) and resuspended in KPS. The parental strains were incubated separately with fusion buffer as above and parents were mixed, without the PEG/$CaCl_2$ incubation, as controls.

Protoplasts were plated in molten potato dextrose agar (Difco potato dextrose broth [PDB] with 12 g l^{-1} agar) containing 0.6 M sorbitol (SPDA) to determine viability. SPDA with selective fungicides (20 μg ml^{-1} propiconazole and either 12 μg ml^{-1} fludioxonil or 45 μg ml^{-1} gliotoxin) was used to detect fusants since, in previous experiments, strain G6 of *T. virens* would not grow on 20 μg ml^{-1} propiconazole and strain Tk7 of *T. koningii* would not grow on either 12 μg ml^{-1} fludioxonil or 45 μg ml^{-1} of gliotoxin (Howell, unpubl.). Protoplasts of both parental strains and mixed strains also were plated on the SPDA with the individual fungicides to confirm lack of growth of parental strain. Plates were allowed to solidify, incubated at 28 °, and examined every 2 d for fungal growth. Fungal colonies from selective medium were transferred to fresh PDA containing selective fungicides. After 5 d, putative fusants were transferred by hyphal tipping to fresh selective medium. Hyphal plugs of putative fusants were transferred five consecutive times on fresh selective medium to test for stability of putative fusants, and then transferred to PDA. After 5 d, agar plugs from the edges of growing colonies were again placed on selective media to test for loss of tolerance to the selective agents. At all transfers, plugs of the parental strains also were transferred from PDA to selective media to confirm inhibition. After transfer to PDA, parental and fusant strains also were plated on PDA containing the individual fungicides to test sensitivity to the individual fungicides as well as the combination.

Culture morphology

Isolates were characterized morphologically by growing them on malt extract agar (Blakslee formula, MEA) (Tuite 1969) in sunlight at 26 ° for 7 d. Isolates were identified according to Domsch, Gams & Anderson (1980), except that *Gliocladium virens* is now called *Trichoderma virens* (Rehner & Samuels 1994).

Secondary metabolite production

The production of secondary metabolites was examined by shake culturing isolates for 6 d in Raulin-Thom medium (RT) (Raper & Thom 1949) or in PDB (Difco), three replicate cultures per isolate. Cultures were filtered through Whatman No. 1 filter paper and the filtrate was extracted with an equal volume of chloroform. The chloroform fraction was evaporated to dryness with a rotary evaporator and the residue was dissolved in methanol. Extracts were stored at −20 °. The pellets were air-dried, and the dry weights were taken to compare growth rates of the isolates.

Inhibitory activity of the extracts was assayed against *Bacillus subtilis*. Twenty μl lots of culture filtrate extracts in methanol were placed in 7 mm-diameter wells in PDA plates, three replicate wells for each extract. The methanol was allowed to vaporize, and dry granular preparations of *B. subtilis* (Gustafson) were shaken over the plates to produce a bacterial lawn. The plates were incubated at 28 °. Controls were chloroform extracts of PDB, RT and methanol alone. Inhibition was measured as the average diameter of the clear zone with no bacterial growth around the well after 48 h. Extraction and antibiotic activity experiments were repeated three times.

For antifungal activity, culture filtrates were applied as above and *Pythium ultimum* or *Rhizoctonia solani* were applied as hyphal plugs (6 mm diam) to the center of the plate. Plates were incubated at 28 ° and examined for inhibition. Inhibition was calculated as the reduction in growth towards wells containing the extract compared to wells with control material after 2 d. Antifungal activity experiments were repeated twice.

To determine the secondary metabolites produced and test for the production of known metabolites from *T. virens*, chloroform extracts in methanol were subjected to high-performance liquid chromatography (HPLC) according to the method of Howell *et al.* (1993).

Randomly amplified polymorphic DNA (RAPD) analysis

Freeze-dried ground mycelium (50 mg) of the *Trichoderma* strains was suspended in lysis buffer (50 mM EDTA, 50 mM Tris, pH 8.0, 3% SDS). Mycelium was incubated at 65 ° for 1 h, frozen at −20 ° for 30 min, and thawed at 65 ° for 10 min. Lysed material was centrifuged in a microcentrifuge (Epindorf) at 12000 ***g*** for 5 min at 7 ° and the supernatant incubated at 24 ° for 30 min with RNAseA (Sigma). Total DNA was extracted by phenol:chloroform extraction followed by ethanol precipitation (Maniatis, Fritsch & Sambrook 1982). Random primers of 10 nucleotides each, as well as M13 universal primers (−20) and (−40) and M13 reverse primer (−24) were obtained from Operon Technologies (Alameda, CA). Sequences of these primers are A03 = AGTCAGCC-AC, B01 = GTTTCGCTCC, B07 = GGTGACGCA-G, C02 = GTGAGGCGTC, C07 = GTCCCGACGA, D07 = TTGGCACGGG, G19 = GTCAGGGCAA, H03 = AGACGTCCAC, H15 = AATGGCGCAG, I20 = AAAGTGCGGG, M13 (−20) = GTAAAAC-GACGGCCAGT, M13 (−40) = GTTTTCCCAGTC-ACGAC, and M13 reverse = AACAGCTATGACC-ATG. Amplification reactions were performed in 25 μl containing 2.5 μl of 10×PCR buffer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM primer, 0.5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN), 50 ng of genomic DNA and $MgCl_2$, with 3.5 μl $MgCl_2$ (25 mM) used with primers B07, C02, C07, G19, H15, and I20, and 2.5 μl used with all other primers. Magnesium chloride levels used were based on research by Y. H. Park (unpubl.). The amplifications were performed in two different thermal cyclers (Perkin Elmer, Foster City, CA, and Ericomp, San Diego, CA) programmed for one cycle of denaturation at 95 ° (2 min) and 45 cycles of 94 ° for 15 s, 40 ° for 30 s and 72 ° for 90 s, followed by 5 min at 72 °. The products were analyzed by electrophoresis in 1.2% agarose gels using tris-borate-EDTA buffer (Maniatis *et al.* 1982) and detected by staining with ethidium bromide. Size markers were obtained from Life Technologies (Gaithersburg, MD). All amplifications were done in duplicate and were done at least once on each thermal cycler.

Biocontrol assay

Biocontrol activity of the *Trichoderma* isolates was tested by growing the isolates in shake cultures of 100 ml of 5% wheat bran and 1% peat moss (WBPM, pH 4.0) as described by Howell *et al.* (1997). After 7 d incubation at 150 rpm, and 26 °, the culture contents were centrifuged at 3000 ***g*** for 10 min at 28 ° and the supernatant fluids were discarded. The solid fractions were air dried for 24 h under a laminar flow hood, ground to a fine powder and stored at 4 °. WBPM preparations were applied with a latex sticker (Rhoplex B-15J, Rohm & Haas, Philadelphia) to cotton seed (*Gossypium hirsutum* cvs 'Stoneville 213' and 'DeltaPine 50') at 0.01 g $seed^{-1}$ and the seeds were planted in flats containing non-sterile field soil. Ten seeds were planted per row and rows were infested with *Rhizoctonia solani* (AG-4) in ground millet as described by Howell *et al.* (1993). After 10 d at 24 °, 14 h days and 20 °, 10 nights, the average survival of seedlings as a percent of the seeds planted was determined for the different *Trichoderma* strains. Controls included seeds coated with latex sticker and the carrier, with and without added *R. solani*. Three replicates were done for each treatment and the biocontrol experiments were repeated three times. Viability of WBPM preparations was determined by plating 10^{-5} dilutions of the air-dried and ground material on PDA. Plates were incubated at 24 ° and examined for fungal growth.

To examine storage quality, dried WBPM preparations of the strains were divided into two lots. The first lot of each preparation was stored at 40 ° and tested every month for 5 months for maintenance of viability and biocontrol activity as described above. Storage at 40 ° was used because this temperature is used in industry to test seed treatments (D. Kenney, pers. comm.). In industrial tests, maintenance of activity of seed treatments stored for one month at 40 ° is considered comparable to maintenance of activity during storage at room termperature for 6 months (D. Kenney, pers. comm.). The second lot was stored for 1 yr at 26 ° and tested every 2 months for viability and biocontrol efficacy as described.

Statistics

Analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) tests were performed using SAS (SAS Institute, version 6, Cary, NC); *t*-tests were performed using Excel (Microsoft, Bothell, WA).

RESULTS

Fusant selection

Sixty colonies were found on SPDA plates containing selective fungicides from the fusant treatments. No colonies were found on selective media from either the mixed parental types without PEG/$CaCl_2$ or from the parental types incubated alone in the fusion buffer. Plates without selective fungicides were completely covered with fungal colonies from all treatments, with colonies of both parental types visible on plates from mixed cultures, either with or without PEG treatment, indicating that protoplasts of both species were viable. Protoplasts of G6 grew on plates containing fludioxonil and gliotoxin, but not on plates containing propiconazole or on the mixed fungicides, and protoplasts of

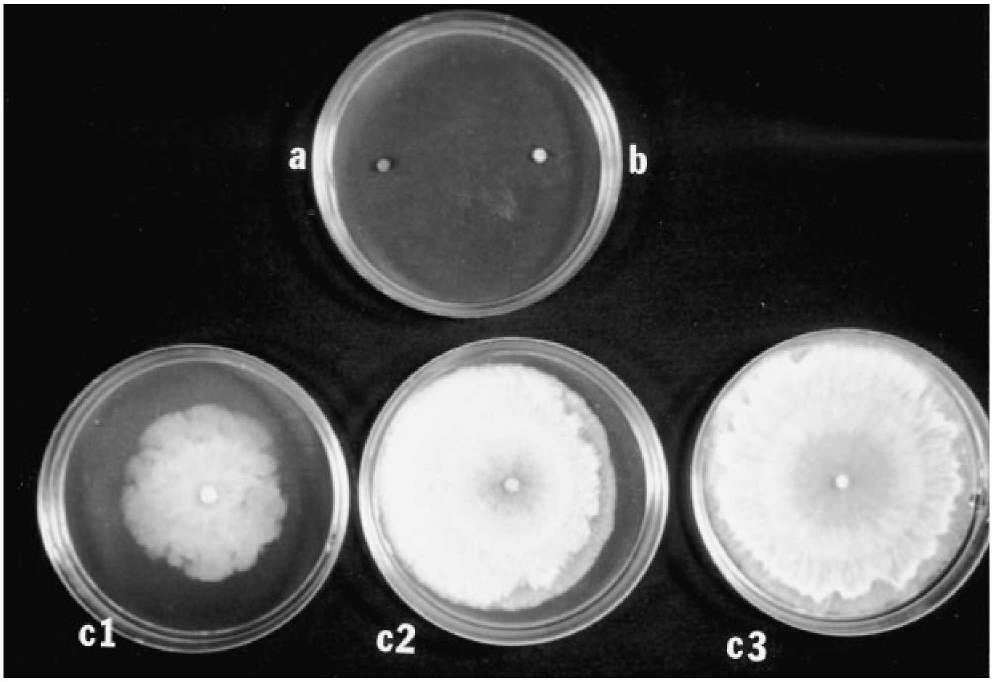

Fig. 1. Growth response of *Trichoderma* isolates to selective agents in PDA. a, *T. virens* parental strain G6; b, *T. koningii* parental strain Tk7; c1, c2 and c3, fusant strains TkG4, TkG12, and TkG18, respectively. The medium contains the fungicides fludioxonil and propiconazole as growth inhibitors.

Table 1. Putative fusants[a], morphology, and media used to select.

Fusant	Morphology	Selective agents
TkG4	*T. koningii*	fludioxonil + propiconazole
TkG12	*T. koningii*	fludioxonil + propiconazole
TkG18	*T. koningii*	fludioxonil + propiconazole
GTk53	*T. virens*	gliotoxin + propiconazole
GTk56	*T. virens*	gliotoxin + propiconazole

[a] Parental strains were *Trichoderma virens* strain G6 and *T. koningii* strain Tk7.

Tk7 grew on plates containing propiconazole, but not on plates containing fludioxonil, gliotoxin, or fungicide combinations.

From the 60 initial colonies on selective SPDA, five isolates were maintained through the initial five transfers. These isolates retained the ability to grow on the selective medium after transfer to non-selective media. Single spore cultures from these isolates also retained the ability to grow on the selective medium, on which the parental isolates could not grow (Fig. 1). The five selected isolates were able to grow on PDA containing the individual selective fungicides. Parental isolates did not show altered responses to the selective media or to the individual fungicides after repeated exposure. Therefore, the isolates obtained were identified as putative fusants.

Culture morphology

On MEA, three of the putative fusants were morphologically similar to Tk7 and were identified as *Trichoderma koningii* (Table 1). All three were obtained from the propiconazole + fludioxonil selective medium. The remaining two isolates were morphologically similar to G6 and were identified as *T. virens* (Table 1). Both of these isolates were obtained from the propiconazole

Table 2. Effect of chloroform soluble materials from *Trichoderma* culture filtrates on growth of *Bacillus subtillis* and *Rhizoctonia solani* on potato dextrose agar.

Treatment	*Bacillus* Inhibition[a]	*Rhizoctonia* inhibition[b]
Methanol	ND	ND
Raulin-Thom medium (RT)	ND	ND
Potato dextrose broth (PDB)	ND	ND
Tk7 (RT)	+	ND
Tk7 (PDB)	+ +	+
TkG4 (RT)	ND	ND
TkG4 (PDB)	+	ND
TkG12 (RT)	+	+
TkG12 (PDB)	+ +	+ +
TkG18 (RT)	+ +	+
TkG18 (PDB)	+ +	+
GTk53 (RT)	+*	+**
GTk53 (PDB)	+	ND
GTk56 (RT)	+ +	+ +
GTk56 (PDB)	+	+
G6 (RT)	+ +	+ +
G6 (PDB)	+	+

[a] Inhibition from 20 μl of concentrated extract from culture filtrate, extract from media alone or the methanol carrier alone. Classes are: none detected = no detectable inhibition; + = inhibition zone < 10 mm diam; + + = inhibition zone > 10 mm diam.

[b] Inhibition of fungal growth from 20 μl of concentrated extract from culture filtrate, media, or the carrier alone.

[c] ND, none detected.

* Significantly different from parental strain (G6) in RT, $P = 0.044$

** significantly different from parental strain (G6) in RT, $P = 0.006$.

Table 3. Secondary metabolites produced by *Trichoderma* fusants and parental strains in Raulin-Thom medium.

	Average concentration (μg compound ml⁻¹ of extract)				
Strain[a]	Gliotoxin	Dimethylgliotoxin	Heptelidic acid	Viridiol	Viridin
G6	7.08[b]	0.60	0.05	0.08	0.11
GTk56	7.04	0.61	0.01	0.05	0.07
GTk53	0.13*	0.04*	ND	0.03	> 0.01*
TkG18	ND	ND	ND	ND	ND
TkG12	ND	ND	ND	ND	ND
TkG4	ND	ND	ND	ND	ND
Tk7	ND	ND	ND	ND	ND

[a] G6 = *T. virens* parental strain, Tk7 = *T. koningii* parental strain, all others are fusant strains.
[b] Each number is the average for three replicates. ND, not detectable by HPLC. *, significantly different from G6 by *t*-test, α = 0.05.

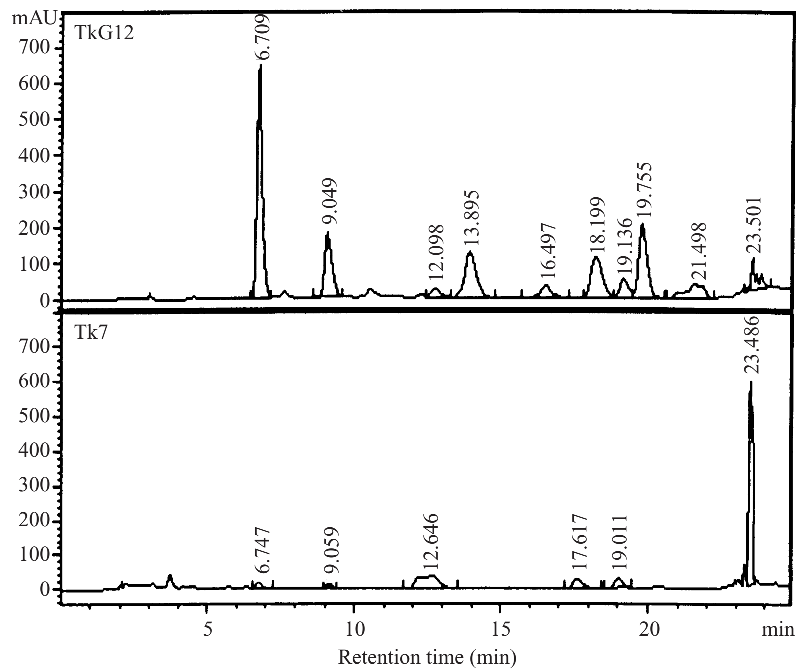

Fig. 2. HPLC chromatograms for chloroform extracts from potato dextrose broth cultures of *Trichoderma koningii* parental strain Tk7 and fusant TkG12. Different peaks, indicating differing compounds, were obtained from the two strains.

+gliotoxin selective medium. On PDA, the *T. koningii* parent, Tk7 and fusant TkG4 did not sporulate in the dark, but fusants TkG12 and TkG18 both sporulated lightly. All of the *T. virens* isolates sporulated readily in the dark.

Secondary metabolite production

Culture filtrate extracts from all of the *Trichoderma koningii* and *T. virens* isolates significantly inhibited the growth of *Bacillus subtilis*, and extracts from at least one medium for all isolates except TkG4 inhibited the growth of *Rhizoctonia solani in vitro* (Table 2). *T. virens* isolates showed greater activity when grown in RT medium, while the *T. koningii* isolates showed greater activity in PDB. The extracts from RT of all of the *T. virens*-type isolates had some inhibitory activity against both *B. subtilis* and *R. solani*, although GTk53 was significantly less inhibitory than the *T. virens* parental strain, G6 ($P = 0.044$ for *B. subtilis*, $P = 0.006$ for *R. solani*). Fusant TkG4 differed from both parental strains in having no detectable inhibitory activity against *R. solani* (Table 2), and less activity than the *T. koningii* parent, Tk7, against *B. subtilis* ($P = 0.003$ for PDB).

When HPLC analysis was performed to compare the levels of secondary metabolites known to be produced by *T. virens*, dimethylgliotoxin, gliotoxin, heptelidic acid, viridin and viridiol, none of the *T. koningii* isolates produced detectable levels of any of these compounds in either RT or PDB. Fusant GTk56 did not differ significantly from the *T. virens* parent in the levels of any of the compounds produced (Table 3), but fusant GTk53 produced significantly less gliotoxin, dimethyl-gliotoxin and viridin, and had no detectable heptelidic

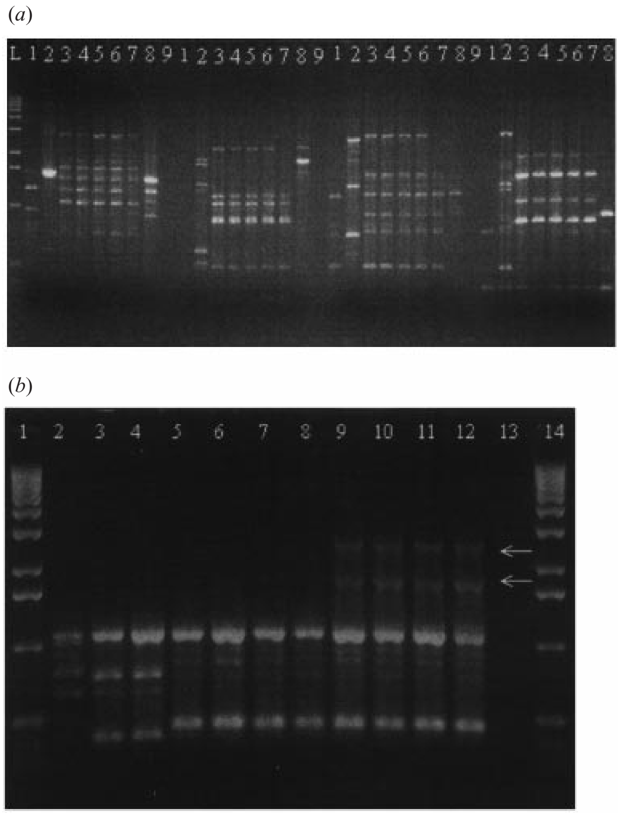

Fig. 3. RAPD banding patterns for parental and fusant strains. (a) *Trichoderma* strains with 4 different primers (H03, H15, G19, and I20 respectively), for each primer, lane 1 – no template control, lane 2 – Tk7, lanes 3–4 GTk56, lanes 5–6 GTk53, lane 7 – G6, lane 8 – *T. harzianum*, lane 9 – no primer control. The first lane (L) contains the 1 kb ladder. (b) *T. koningii* strains with primer M13 (−40). Lanes 1 and 14 – 1 kb ladder, lane 2 – no template control, lanes 3–4 – G6, lanes 5–6 – TkG18, lanes 7–8 – TkG12, lanes 9 & 10 TkG4, lanes 11–12 – Tk7, lane 13 – no primer control. Arrows indicate the two bands that differ between the parental strain, Tk7, and the fusant strains, TkG12 and TkG18.

acid. This was determined to be a difference in production and not secretion by extracting the hyphal material with 80 % acetone (Howell *et al.* 1993). Levels of all of the compounds were also lower in the pellet from GTk53 when compared to the pellet from G6 (data not shown), indicating that secondary metabolites were not being retained at higher levels within the mycelium of GTk53. The growth rate of GTk53, as determined by relative dry weight of the fungal pellet, was not significantly different from that of G6 in RT by *t*-test ($P = 0.29$).

Fusant TkG12 also differed from its morphologically similar parent in secondary metabolite production. As shown by the HPLC chromatograms (Fig. 2), TkG12 produced several peaks that were not detectable in the *T. koningii* parent, Tk7. None of these peaks had a spectrum similar to any of those found in extracts from *T. virens*. Fusant TkG4 had much smaller peaks than did the parental strain, Tk7, although the growth rate was not significantly different by *t*-test ($P = 0.68$).

Table 4. Biocontrol of *Rhizoctonia*-induced damping-off of cotton (cv DeltaPine 50) seedlings with *Trichoderma* parental and fusant strains and the effect of storage at 40 °C on activity.

	Average percent survival of cotton seedlings			
Treatment[a]	0 months[b]	1 month	3 months	5 months
WBPM control	83 a[c]	83 a	90 a	80 a
Tk7	20 cd	3 d	7 ef	3 b
TkG4	13 d	3 d	3 f	3 b
TkG12	40 b	40 b	53 bc	13 b
TkG18	50 b	17 cd	23 def	3 b
GTk53	33 b	13 cd	23 def	0 b
GTk56	83 a	33 bc	43 cd	7 b
G6	80 a	17 cd	33 de	3 b
Rhizoctonia	13 d	0 d	7 ef	0 b

[a] Treatments are seed treated with wheat bran + peat moss preparations of *Trichoderma* strains or the wheat bran + peat moss carrier alone. The WBPM control has no *Rhizoctonia* added while all other treatments have *R. solani*.

[b] Zero months is fresh material, before storage.

[c] Numbers are averages for three replicates. Numbers in each column followed by the same letter are not significantly different by Fisher's least significant difference test ($\alpha = 0.05$).

RAPD analysis

Twelve of the 13 RAPD primers amplified DNA from the *Trichoderma* strains. Primer B01, which had been used to amplify DNA from *Trichoderma* species by Zimand *et al.* (1994), did not produce any detectable amplification from any of our *Trichoderma* strains, either parental or fusants. The RAPD patterns produced by the other 12 primers differed between the *T. koningii* and *T. virens* parental strains. All of the fusants were similar in RAPD banding pattern to the morphologically similar parent. An example of the similarity in banding patterns between the fusants and one parental strain is shown in Fig. 3a with four primers, H03, H15, G19 and I20. No bands were observed in the template DNA controls without primers. For six of the 13 primers, bands were detected when no template DNA was added. This is consistent with the report of Pan *et al.* (1997), in which nonspecific bands were found in the absence of template DNA from a number of RAPD primers. The banding patterns in the absence of template DNA were not identical to those from either *Trichoderma* species. With only one primer, M13 (−40) was a reproducible difference detected between any of the fusants and the morphologically similar parent. Two bands were absent in the fusant TkG12 and about half as intense in TkG18 compared to bands of the same size from the parental strain, Tk7, and fusant TkG4 (Fig. 3b).

Biocontrol assays

In biocontrol assays, three of the putative fusants initially differed significantly in activity from the parent to which they were morphologically similar (Table 4). TkG12 and TkG18 gave significantly greater control of *Rhizoctonia solani* than did the *Trichoderma koningii*

Table 5. Biocontrol of *Rhizoctonia solani*-induced damping-off of cotton seedlings (cv. DeltaPine 50) by *Trichoderma* parental and fusant strains with storage at 26 °C.

	Average percent survival of cotton seedlings			
Treatment[a]	0 months[b]	6 months	8 months	12 months
WBPM control	83 a[c]	100 a	97 a	90 a
Tk7	20 cd	23 d	10 ef	7 d
TkG4	13 d	17 d	7 f	7 d
TkG12	40 b	50 bc	40 bc	33 b
TkG18	50 b	30 cd	30 cde	17 cd
GTk53	33 b	23 d	20 def	13 cd
GTk56	83 a	73 b	53 b	23 bc
G6	80 a	53 bc	37 bcd	20 bcd
Rhizoctonia	13 d	20 d	17 def	13 cd

[a] Treatments are seed treated with wheat bran + peat moss preparations of *Trichoderma* strains or the wheat bran + peat moss carrier alone. The WBPM control has no *Rhizoctonia* added while all other treatments have *R. solani*.

[b] Zero months is fresh material, before storage.

[c] Numbers are averages for three replicates. Numbers in each column followed by the same letter are not significantly different by Fisher's least significant difference test ($\alpha = 0.05$).

parent, and GTk53 gave significantly less control that did the *T. virens* parent. The *T. virens* parental strain had lost the majority of its activity by the end of the first month of storage at 40 °. However, fusant TkG12 maintained control activity against *R. solani* for up to 3 months of storage at 40 °, at which time the *T. virens* strains with biocontrol activity and strain TkG18 all showed significant reductions in efficacy (Table 4). Similarly, strain TkG12 maintained biocontrol efficacy (significantly higher survival than the carrier control) after storage 1 yr at room temperature, at which time the other strains showed significantly reduced biocontrol activity (Table 5). Fusant GTk56 had a slower rate of activity loss than the other *T. virens* strains. When stored at 40 °, GTk56 gave survival rates that were significantly higher than the pathogen control after both 1 and 3 months and it gave survival rates after 8 months at 26 ° that were significantly higher than those of the pathogen treatment alone, while none of the other *T. virens* strains were significantly different from the *Rhizoctonia* control at any of these times.

All of the air-dried materials contained viable fungal propagules throughout the duration of the study. However, germination from stored materials was somewhat slower than that from fresh material. With fresh WBPM preparations, fungal growth was visible without a dissecting scope within 24 h after plating, but with stored materials, growth was not visible without magnification until at least 48 h after plating except for strains Tk7 and TkG12, which showed growth within 24 h after plating at all times tested.

DISCUSSION

A larger number of protoplasts without exposure to fusion conditions were tested for growth on the selective agents than those with fusion treatment (the parental strains alone, with and without PEG treatment, and the combined strains without PEG treatment, resulting in at least three times as many non-fused protoplasts of each type). Thus, if the altered resistance were due to variability in the protoplasts, we would expect some protoplasts or single spores to grow on the selective media in the absence of the fusion treatment. Since this was never observed it is unlikely that our isolates were obtained due to inherent genetic variability and we conclude that they are most likely fusants.

The similarity of all five fusants to one of the parental isolates is consistent with the results of previous researchers. For example, in fusions reported between *Trichoderma virens* and *T. harzianum*, 17 out of 24 stable strains formed colonies similar to those of *T. virens* (Shin & Cho 1993). Similarly, in an intergeneric fusion between *T. longibrachiatum* and *Phanerochaete chrysosporium*, the fusant obtained was phenotypically similar to the *T. longibrachiatum* parent and quite dissimilar to the *P. chrysosporium* parent (Nutsubidze *et al.* 1991). This fusant was reported to differ from the *T. longibrachiatum* parent in pigment production, sporulation, growth rate, and enzymatic activity (Nutsubidze *et al.* 1991). This is consistent with the differences we observed in our fusants which, while generally phenotypically similar to one parent, differed in characters such as sporulation and secondary metabolite production. Progeny from other protoplast fusions have been reported to differ from the parental strains in characteristics such as pigmentation (Shin & Cho 1993, Kumari & Panda 1994), secondary metabolite production (Kumari & Panda 1994) and nutritional status (Stasz *et al.* 1989, Kumari & Panda 1994).

Our results with RAPDs indicate that the fusants are genetically similar to the phenotypically similar parent. This is consistent with results of Stasz & Harman (1990) for isozyme banding patterns from stable progeny from protoplast fusions, in which only parental isozyme patterns were obtained, although fusants differed in several other characteristics.

Variability in secondary metabolite production has been reported in the progeny from other protoplast fusions (Nutsubidze *et al.* 1991, Kumari & Panda 1994). This variability in secondary metabolite production may be important in biocontrol of diseases other than those caused by *Rhizoctonia solani*. Studies with UV mutants of *T. virens* have indicated that antibiotic production is important for control of *Pythium ultimum* (Howell & Stipanovic 1983, Wilhite, Lumsden & Straney 1994), but not *R. solani* (Howell & Stipanovic 1995). The reduction in gliotoxin production in fusant GTk53 probably is not the reason for the reduced biocontrol activity, since mutants that have completely lost gliotoxin production are still effective biocontrol agents of *R. solani*-incited cotton seedling disease (Howell & Stipanovic 1995). This strain will be used in studies to examine other factors that might be involved in biocontrol activity.

For the *T. koningii* fusants, the variability in secondary metabolite production is being examined further. The parental strain and fusants TkG12 and TkG18 all produce the antibiotic koninginin G, based on comparison to a known standard by HPLC and thin layer chromatography, but antibiotic activity also is detectable from other fractions of the extract of TkG12. The compounds produced by fusant strain TkG12 that are not detectable in the parental strains will be identified.

Variability in biocontrol activity similar to what we observed has been reported in protoplast fusants of other *Trichoderma* species (Pe'er & Chet 1990). In comparison with the morphologically similar parent, we found both strains with increased biocontrol activity (TkG12 and TkG18) and decreased biocontrol activity (GTk53). The level of biocontrol activity of fusant GTk53, while significantly lower than that of the morphologically similar *T. virens* parent, G6, still was a significant level of biocontrol activity, giving seedling survival rates similar to those of the two improved *T. koningii* strains.

The maintenance of activity in two fusants, GTk56 and TkG12 indicates that we were able to successfully combine some biocontrol activity from one parent with storage quality from the other parent. While fusant GTk56 had a higher initial activity than did TkG12 and maintained activity for longer than did the *T. virens* parental strain, it had a significant loss of activity compared to that obtained with fresh material at all storage times tested (Tables 4–5). This combined with the loss of significant difference in survival after a year of storage at 26 °, indicated that GTk56 did not maintain activity as well as TkG12. Further work will be done to better combine high levels of biocontrol activity with good maintenance of activity following storage.

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